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Technical Note

Detection of Endogenous Alkaline Phosphatase Activity in Intact Cells by Flow Cytometry Using the Fluorogenic ELF-97 Phosphatase Substrate

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Background: The alkaline phosphatase (AP) substrate 2-(5′-chloro-2′-phosphoryloxyphenyl)-6-chloro-4-(3H)-quinazolinone (ELF®-97 for enzyme-labeled fluorescence) has been found useful for the histochemical detection of endogenous AP activity and AP-tagged proteins and oligonucleotide probes. In this study, we evaluated its effectiveness at detecting endogenous AP activity by flow cytometry.

Methods: The ELF-97 phosphatase substrate was used to detect endogenous AP activity in UMR-106 rat osteosarcoma cells and primary cultures of chick chondrocytes. Cells were labeled with the ELF-97 reagent and analyzed by flow cytometry using an argon ultraviolet (UV) laser. For comparison purposes, cells were also assayed for AP using a Fast Red Violet LB azo dye assay previously described for use in detecting AP activity by flow cytometry.

Results: The ELF-97 phosphatase substrate effectively detected endogenous AP activity in UMR-106 cells, with over 95% of the resulting fluorescent signal resulting from AP-specific activity (as determined by levamisole inhibition of AP activity). In contrast, less than 70% of the fluorescent signal from the Fast Red Violet LB (FRV) assay was AP-dependent, reflecting the high intrinsic fluorescence of the unreacted components. The ELF-97 phosphatase assay was also able to detect very low AP activity in chick chondrocytes that was undetectable by the azo dye method.

Conclusions: The ELF-97 phosphatase assay was able to detect endogenous AP activity in fixed mammalian and avian cells by flow cytometry with superior sensitivity to previously described assays. This work also shows the applicability of ELF-97 to flow cytometry, supplementing its previously demonstrated histochemical applications. Cytometry 37:314–319, 1999. © 1999 Wiley-Liss, Inc.

Key terms: fluorescence; flow cytometry; alkaline phosphatase; ELF-97; Fast Red Violet LB

The synthesis and identification of fluorogenic substrates for common enzymes has accelerated in recent years, both for analysis of enzyme activity in lysates and intact cells, and as detectable fluorescent tags for enzyme-labeled proteins and nucleic acids. Development of fluorescent substrates for alkaline phosphatase (AP) has been particularly productive. Several fluorescein- and coumarin-based and other substrates have been synthesized and used for the detection of endogenous AP activity in whole cells and lysates and for fluorescent tagging procedures (1–5). In general, these substrates are essentially nonfluorescent when their phosphate group is attached, but fluoresce brightly upon cleavage in the presence of the appropriate mercury-arc lamp or laser excitation.

Although these substrates have been found useful for a number of applications including microplate-based AP assays and in-gel labeling of AP-labeled nucleic acids, they have been less useful in techniques involving the labeling of intact cells and tissues for fluorescence or confocal laser scanning microscopy and flow cytometry. Most of these substrates remain water-soluble before and after phosphate cleavage, preventing their hydrolysis products from remaining localized in intact cells and tissues, and limiting

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their usefulness in these applications (3,6). Several AP assays have been described involving the cleavage of a naphtholic phosphate AP substrate (under low-fluorescing, precipitating conditions) which subsequently reacts with a diazonium salt, resulting in an insoluble, fluorescent azo dye adduct (3,7–11). This adduct forms a precipitate at the reaction site that can be detected colorimetrically or by its fluorescence (3). These azo dye or ''capture'' systems have been used for the detection of both endogenous and tagged AP activity in cells, tissues, and chromosomes (12–15). Several of these assays have been adapted to the flow cytometric detection of AP activity, including AS-BI and AS-MX substrates with Fast Blue BB, Fast Red TR, or Fast Red Violet LB diazonium salts (8–10,13,16). The Fast Red Violet LB (FRV) assay has been shown to be particularly AP-specific with low background fluorescence relative to other azo dye assays (12). Although widely used, these capture assays share a number of disadvantages, the greatest being the high intrinsic fluorescence of the unreacted assay components, making for a low signal-to-noise ratio and low sensitivity (3,9,17). The need therefore exists for a substrate that is nonfluorescent and water-soluble prior to cleavage but that directly forms a fluorescent insoluble precipitate upon cleavage at the site of enzymatic activity (6). This substrate would also need to be excited by wavelengths commonly found in fluorescence microscopy and/or flow cytometry.

Recently, Singer et al. (18) described the development of the fluorogenic AP substrate 2-(5′-chloro-2′-phosphoryloxyphenyl)-6-chloro-4-(3H)-quinazolinone (termed ELF-97 phosphate). This substrate is water-soluble and essentially nonfluorescent in its unreacted state, but forms an ultraviolet (UV)-excited, highly fluorescent yellow-green precipitate upon AP cleavage. This substrate has been used successfully in a number of histochemical applications, including measurement of endogenous AP, labeling of EGF receptors, detection of endogenous AP in zebrafish retinal tissues, and localization of AP-transfected cells in regenerating newt limbs (17–20). ELF-97 phosphate has also been used to detect AP-conjugated probes hybridized to chromosomes, mRNAs in intact cells and tissue sections, and DNA in gels and on filters (21,22). Since the substrate is optimally excited in the UV range obtainable from both argon- and krypton-ion lasers, it is likely that it will function well in flow cytometric assays as well. A recent report described a method for using the ELF-97 phosphate to measure AP activity in samples of marine phytoplankton by flow cytometry (23). In this paper, we report on the use of this substrate to detect endogenous AP activity in avian and mammalian cells by multilaser flow cytometry.

MATERIALS AND METHODS

Cell Culture and Fixation

UMR-106 cells obtained from the American Type Culture Collection (Manassas, VA) were maintained in D-MEM with 10% fetal bovine serum (FBS) and trypsinized prior to passage. Mesenchymal cells were removed from the femurs and tibias from chick embryos, spotted onto plastic dishes, and cultured in RPMI with 20% FBS and 5% chicken serum, with media changes every 3 days until the cells differentiated into chondrocytes. For both ELF-97 and Fast Red Violet LB (FRV) assays, UMR-106 cells were removed from culture dishes by trypsinization, filtered through nylon mesh to generate single-cell suspensions, and treated with 70% EtOH at 4°C for up to 24 h. Chick chondrocytes were fixed in 70% EtOH while still adhering to their culture dishes, washed multiple times with normal saline (0.15 M NaCl), and removed by gentle scraping and filtered through nylon mesh as above.

ELF-97 Alkaline Phosphatase Assay

Cells were washed twice by centrifugation with normal saline (0.15 M NaCl) without phosphate buffers to remove ethanol. Cells were then preincubated in the ELF-97 developing buffer provided by the manufacturer without or with levamisole at 5 mM for 15 min. Prior to use, the ELF-97 phosphate was filtered through a 0.2-μm centrifuge filter to remove precipitated substrate. The ELF-97 phosphate was then added to the cells in a final volume of 100 μl at the final concentration indicated by the manufacturer (1:20 in the provided buffer). Cells were incubated at room temperature for times ranging from 1–30 min, followed by the addition of levamisole at 10 mM to all tubes to stop the reaction. The cells were then analyzed by flow cytometry within 1 h of reaction completion.

Fast Red Violet LB Alkaline Phosphatase Assay

The Fast Red Violet LB (FRV) assay was carried out as previously described, with minor modifications (16). Cells were washed as above to remove ethanol and preincubated in a reaction buffer containing 0.1 M Tris with 0.5% DMF, pH 9.0, without or with levamisole as described above. Cells were then incubated in Fast Red Violet LB at 0.1 mg/ml and naphthol AS-BI phosphate at 0.03% final concentrations in the above reaction buffer for timepoints ranging from 1–30 min. For some control samples, naphthol AS-BI phosphate was omitted from the reaction. The reaction was stopped with levamisole as described above, and analyzed by flow cytometry within 1 h.

Flow Cytometry

Cells were analyzed on a Becton Dickinson FACSVantage (San Jose, CA) using a Coherent Enterprise 921 argon-ion laser emitting simultaneously at 488 nm (150 mW) and 351 nm (50 mW). Cell forward and side scatter were measured using the 488-nm beam. ELF-97 alcohol precipitate fluorescence was excited using the spatially separated 351-nm UV beam and the signal reflected with a 640-nm long pass reflecting dichroic and measured through a 530 ± 30-nm narrow bandpass filter. FRV assay fluorescence was excited using the 488-nm beam and the resulting signal reflected with a 610-nm short pass dichroic and measured through a 675 ± 20-nm narrow bandpass filter. ELF-97 alcohol precipitate and FRV adduct fluorescence were expressed as the mean fluorescence intensity (MFI) of the total sample fluorescence compared with

both unlabeled and levamisole-inhibited control samples. Assays were compared by calculating the fold increase in fluorescence of total sample signal divided by the levamisole-inhibited control values.

Fluorescence Microscopy

The ELF-97 reaction was carried out essentially as described for flow cytometry on 30-mm dishes containing either ethanol-treated UMR-106 cells or chick chondrocytes. Cells were visualized and photographed on a Nikon Diaphot fluorescence microscope using a standard Hoechst/DAPI longpass filter set. Photomicrography was carried out using a Sony DKC-5000 CatsEye CCD camera and image acquisition system.

Reagents

The ELF-97 phosphatase assay was performed using the ELF-97 Endogenous Phosphatase Detection Kit (E-6601) provided by Molecular Probes, Inc. (Eugene, OR). FRV assay reagents were obtained from Sigma Chemical Co. (St. Louis, MO). All tissue culture reagents were obtained from Life Technologies (Gaithersburg, MD).

RESULTS AND DISCUSSION

To determine if the ELF-97 phosphate was useful for detecting endogenous AP in intact cells, we utilized the osteosarcoma cell line UMR-106, notable for its unusually high level of endogenous AP expression, and in vitro cultured chick chondrocytes, which demonstrate more ''normal'' levels of endogenous AP activity. Figure 1 shows fluorescence micrographs of UMR-106 cells assayed with ELF-97 phosphatase substrate in the absence (Fig. 1a) or presence (Fig. 1b) of the AP inhibitor levamisole. Figure 1a shows the characteristic bright green fluorescence and fine-grained precipitate of the ELF-97 reaction product. We then evaluated the usefulness of the ELF-97 phosphatase assay for flow cytometric analysis based on its overall ''brightness,'' using the laser excitation sources and emission filters and dichroics commonly found in commercially available fluorescence-activated cell sorters and on the background fluorescence level of the unreacted substrate. We also compared the assay to the most commonly used system for detecting AP activity in cells by flow, namely a Fast Red Violet azo dye assay described previously (16).

Figure 2 shows a reaction time-course from 1–30 min, with the background fluorescence level of no reagents added, levamisole alone, ELF-97 phosphate alone, and ELF-97 phosphate plus levamisole samples shown. In this experiment, the cells were stimulated with a relatively low-power argon-ion UV laser (Coherent Enterprise 921 emitting at 50 mW) and detected through a standard fluorescein filter (530 ± 30 nm). Conversion of the substrate to the insoluble fluorescent ELF-97 alcohol was extremely rapid, showing a strong fluorescent signal in as little as 1 min. Even at this early time point, the AP-specific fluorescence (the difference between the fluorescence detected in the absence and presence of levamisole) accounted for almost 90% of the total fluorescent signal. By 30 min, AP-specific fluorescence accounted for greater

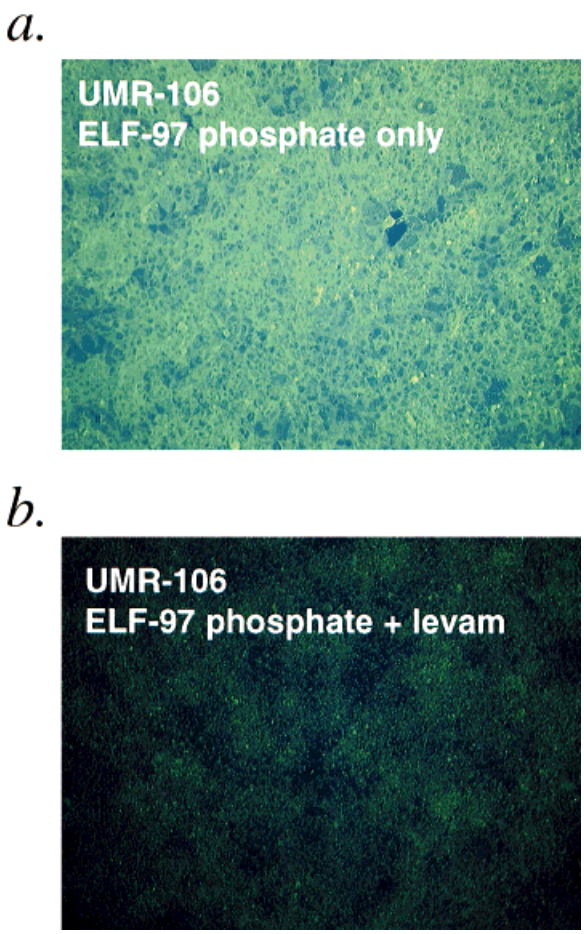

FIG. 1. Fluorescence micrographs of UMR-106 rat osteosarcoma cell monolayers labeled with the ELF-97 phosphatase substrate in the absence (**a**) or presence (**b**) of the AP inhibitor levamisole at 5 μM. Cells were visualized on a Nikon Diaphot fluorescence microscope with a 10× objective and a standard Hoechst/DAPI longpass filter set, and digitally photographed using a Sony DKC-5000 CatsEye CCD camera and image acquisition system.

than 98% of the total signal. These results show that the ELF-97 phosphatase substrate produced a strong signal in the presence of endogenous AP, with extremely low background and a large signal-to-noise ratio. Excitation of the cells with other standard UV laser sources (such as a Coherent I-90 krypton-ion laser emitting at 351 nm) gave comparable results (data not shown).

The results obtained from the ELF-97 phosphatase assay were then compared to those from an FRV azo dye assay for AP activity, as previously described (12). A reaction time course is shown in Figure 3. At 1 min, the AP-specific signal of the FRV adduct constituted less than 70% the total AP-specific fluorescence. By 30 min, this value was still at approximately 80% of the total signal. While this constituted a usable assay, these results illustrated that the Fast Red Violet LB possessed considerable fluorescence in its unreacted state, complicating detection of AP activity and reducing its sensitivity. The extremely low fluorescence of the unreacted ELF-97 phosphate resulted in a

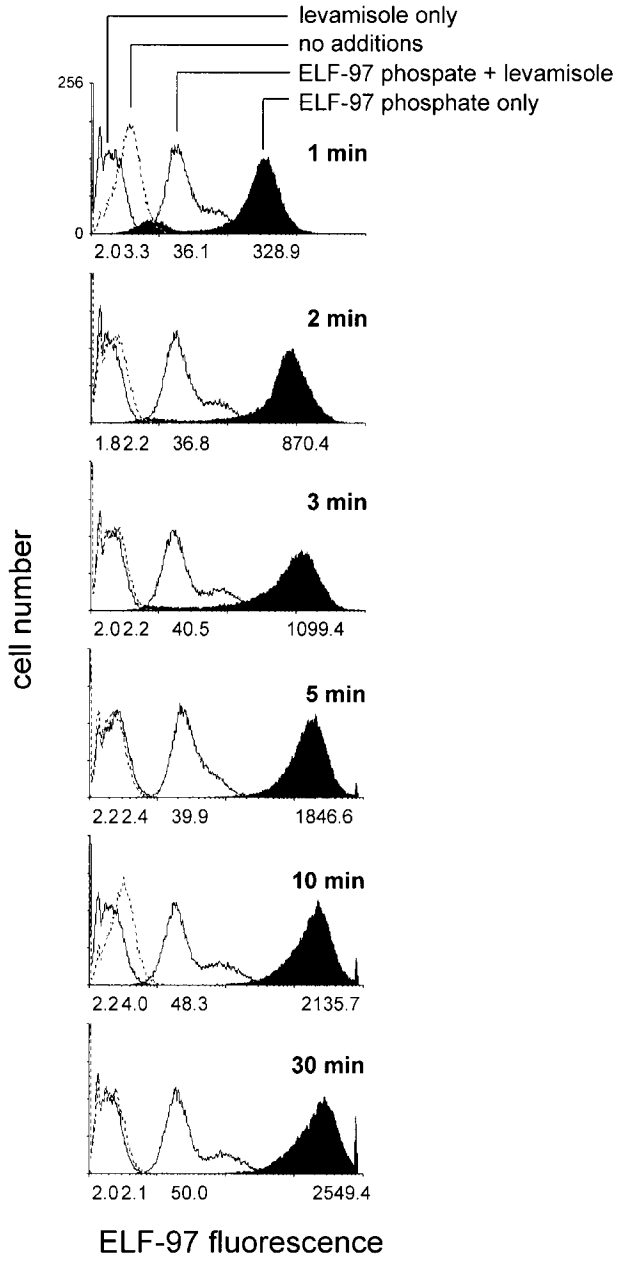

FIG. 2. AP activity in UMR-106 cells, as measured by flow cytometric detection of ELF-97 product fluorescence at time points ranging from 1–30 min. Fluorescence peaks in the absence of any additions, with ELF-97 phosphate only, preincubation with levamisole only (5 mM), and ELF-97 phosphate with levamisole are shown, with the corresponding mean fluorescence intensity values for each peak indicated below the x-axis.

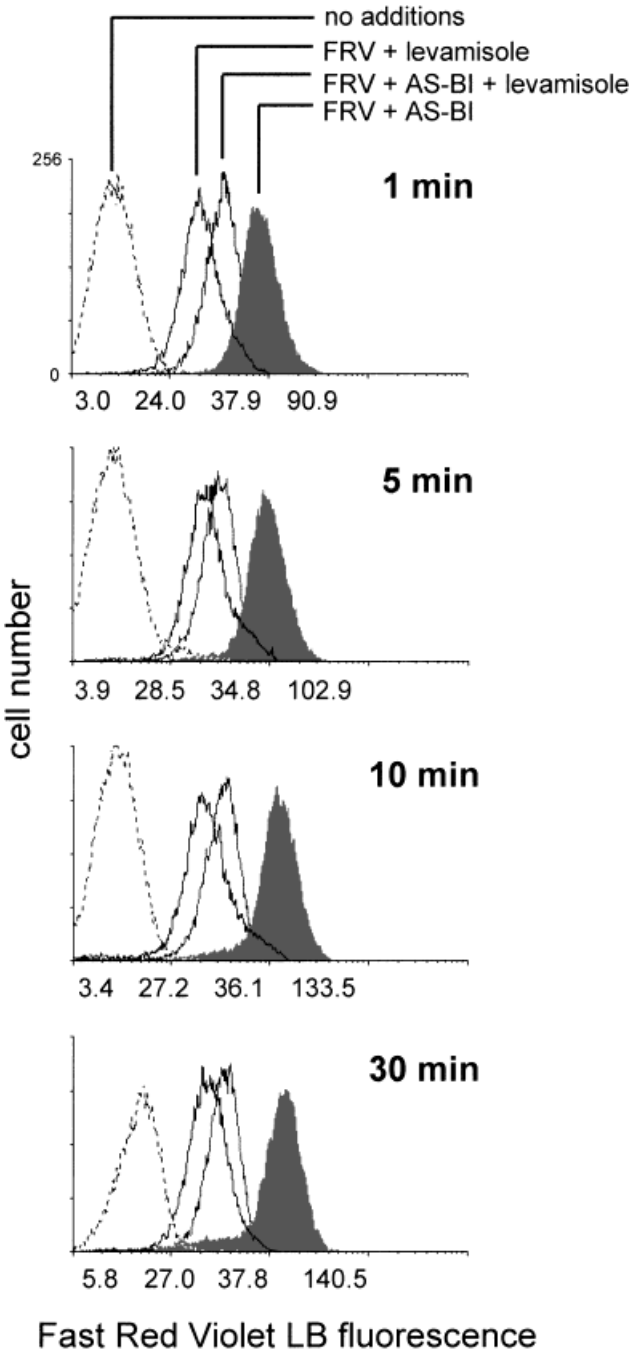

FIG. 3. AP activity in UMR-106 cells as measured by flow cytometric detection of FRV adduct fluorescence at time points ranging from 1–30 min. Fluorescence peaks in the absence of any additions, with FRV/substrate only, levamisole only, and FRV/substrate with levamisole are shown, with the corresponding mean fluorescence intensity values for each peak indicated below the x-axis.-

high signal-to-noise ratio, enhancing the assay's sensitivity. Signal-to-noise ratios for the ELF-97 and FRV assays were greater than 50:1 and less than 5:1, respectively, after 30 min. The ELF-97 phosphatase assay therefore showed approximately a 10-fold increase in sensitivity over the FRV assay.

From a practical standpoint, the FRV assay was also complicated by the large granular precipitate produced during the reaction. This has been previously reported as being detrimental to precise localization of AP activity in tissue (13,18). In the case of flow cytometry, this precipitate interfered with the stream-in-air nozzle flow, complicating sample analysis. The precipitate produced by the ELF-97 phosphatase assay was far less granular and produced no such fluidics problems.

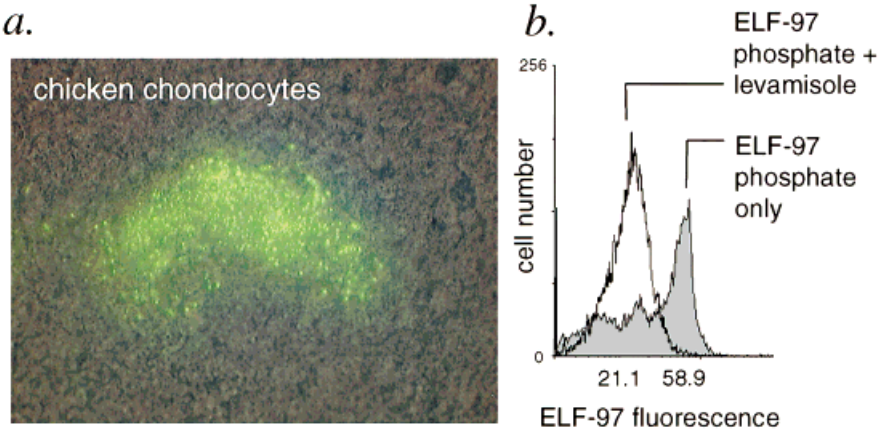

FIG. 4. **a:** Fluorescence micrograph of chick chondrocytes labeled with the ELF-97 phosphatase substrate. Cells were visualized by epifluorescence and brightfield illumination simultaneously, using the system and conditions described in Figure 1. **b:** AP activity in chick chondrocytes, as measured by flow cytometric detection of ELF-97 alcohol product fluorescence. Fluorescence peaks and mean fluorescence intensity values for samples without and with preincubation with levamisole at 5 mM are shown.

UMR-106 cells express abnormally high levels of endogenous AP. To determine if the ELF-97 phosphate assay was useful in detecting AP activity in a nonneoplastic cell system, we labeled in vitro cultured chick chondrocytes on day 26 postplating (no phosphate added). Figure 4a shows a fluorescence micrograph of a chondrocyte colony (on an AP-negative background of immature mesenchymal cells) assayed with ELF-97 phosphatase substrate. The presence of AP activity is demonstrated by the green fluorescence of the ELF-97 alcohol precipitate. This AP activity could also be detected by flow cytometry using the ELF-97 phosphatase assay and the excitation/emission conditions described above (Fig. 4b). In contrast, this low level of AP activity was not detectable by the FRV assay (data not shown).

The ELF-97 phosphatase assay therefore proved to be superior to previous methods of detecting endogenous AP activity by flow cytometry. Although a variety of substrates that become fluorescent following enzyme modification have been explored for flow cytometry, practical assays for AP activity have generally been limited to azo dye methods. Although several such assays have been described in the literature (using the diazonium salts Fast Red, Fast Blue, and Fast Red Violet), the high intrinsic fluorescence of the unreacted components has made these assays of limited value for flow cytometry. Fast Red and Fast Red Violet LB assays have generally been the only ones sensitive enough to measure endogenous AP by flow cytometry, and they are still hampered by high backgrounds and lack of specificity (3,8–10,13,16,17). The ELF-97 phosphate showed negligible fluorescence in the unmodified state and is highly fluorescent following cleavage, showing greater signal-to-noise ratio and much higher sensitivity than the azo dye systems.

One requirement of the ELF-97 phosphatase assay is a UV excitation source, common in fluorescence and confocal laser scanning microscopy, but generally limited to more sophisticated flow cytometers and sorters. Although this precludes use of the ELF-97 phosphatase substrate on single-laser benchtop flow cytometers, azo dye assays also generally require a high-power 488-nm laser source to distinguish AP-specific fluorescence from background (data not shown). The results shown above required a minimum laser output of 150 mW, several times higher than that generated by most air-cooled argon-ion lasers in benchtop instruments. Sensitivity of the FRV assay was considerably reduced when carried out on a benchtop flow cytometer with a 50-mW argon laser (data not shown). Useful fluorochromes that excite in the UV range are also limited in number; the ability to excite the ELF-97 product with the UV beam of a multilaser instrument frees up the detection channels of the primary 488-nm laser beam, opening up possibilities for multicolor analysis incorporating AP detection. The ELF-97 phosphatase assay has been used simultaneously with tetramethylrhodamine, propidium iodide, Cascade Blue, the Hoechst dyes, and others (17,19,22). The ability to detect the ELF-97 product via a UV laser source and its compatibility with other fluorochromes will undoubtedly make it a useful reagent for multicolor, multilaser flow cytometric analysis.

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